Living End of Hope DoD Breast Cancer Research Program
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Abstract

Overexpression and amplification of the Her-2/*neu* proto-oncogene has been implicated in the development of aggressive human breast cancer. Consequently, Her-2/neu provides a potential target for immunotherapy. We have employed a novel *in vivo* gene transfer system based on the Venezuelan Equine Encephalitis (VEE) virus. Our approach has been to establish VEE based replicons encoding Her-2/*neu* as well as pro-inflammatory cytokines known to promote $CD4^+$ Th1 and $CD8^+$ CTL activity. To determine the therapeutic efficacy of the VEE-based replicons, mice transgenic for the rat Her-2/*neu* gene (FVB/*neu*) have been employed. To date we have generated and tested replicons encoding the complete 185 kDa Her-2/neu transmembrane protein, a truncated protein spanning the extracellular and transmembrane domains of Her-2/neu (Neu/ECD-TM) as well as replicons encoding the cytokines IL-2, IL-4, IL-12 and GM-CSF. We have repeatedly generated CTL responses in wild type FVB mice and are currently evaluating strategies to overcome Her-2/neu-specific tolerance in FVB/*neu* mice. Additionally, we have established a Her-2/*neu* expressing tumor cell line derived from FVB/*neu* mice. FVB/*neu* mice are permissive for growth of the tumor cell line making this system amenable to tumor challenge experiments. We have observed delayed or absent tumor growth in FVB/*neu* mice immunized with the truncated VEE Her-2/*neu* construct and are currently evaluating the cellular phenotype responsible for this inhibition. We believe establishing a vaccine protocol using VEE replicons encoding Her-2/*neu* and appropriate cytokines will provide an effective strategy for enhancing tumor antigen-specific $CD4^+$ Th1 and $CD8^+$ CTL reactivity for the purpose of preventing tumor progression and providing long-term protection from tumor recurrence.

Introduction

Tumor antigen-specific immunotherapy has long been thought of as an ideal approach to treating various forms of cancer. In large part this is due to the specificity associated with an immune response resulting in destruction of only tumor antigen bearing cells. Further, eliciting immune function can induce immunological memory, and consequently provide long-term protection from tumor recurrence. However, most tumor associated antigens are in fact self-antigens, and by definition poor immunogens. Consequently, the issue of self-tolerance remains a significant hurdle to overcome for inducing effective tumor specific immunity. One such tumor-associated antigen is encoded by the Her-2/*neu* (*erb*B-2/*neu*) proto-oncogene. Her-2/*neu* encodes a transmembrane protein that is a member of the epidermal growth factor receptor (EGFR) family of proteins. Overexpression and amplification of Her-2/*neu* has been implicated in the development of aggressive breast adenocarcinoma as well as in the development of ovarian cancer. Her-2/*neu* is over-expressed to some degree in 91% of all breast cancers, and higher levels of over-expression correlate with a poor prognosis. Importantly, cytotoxic lymphocytes (CTL) cultured from human breast and ovarian cancer patients have been shown to be specific for a number of peptides derived from the Her-2/neu protein. This suggests tolerance to the self-antigen is incomplete and may provide for a window through which enhanced immune reactivity may be achieved. FVB mice transgenic (Tg) for the Rat Her-2/*neu* proto-oncogene (FVB/*neu*) under the control of the mouse mammary tumor virus (MMTV) 3' LTR promoter/enhancer have been developed and characterized previously. In this strain of mice, 50% of females develop focal mammary tumors surrounded by hyperplastic mammary epithelium by 30 weeks of age. Of these, 70% display a tendency toward pulmonary metastasis by 8 months. Tumor cells removed from these mice display elevated levels of the transgene

bolstering evidence for the oncogenic potential of Her-2/*neu*. $CD4^+$ T helper (Th) cells may be divided into subpopulations based on their cytokine secretion profile and their ability to regulate either a pro-inflammatory (Th1) or anti-inflammatory (Th2) response. Antigen specific $CD4^+$ Th cell activity is typically required for effective CTL responses to poor immunogens and establishment of immune memory. Therefore, a treatment that is able to boost tumor antigen specific $CD4^+$ Th1 cell activity should subsequently increase $CD8^+$ CTL reactivity, and possibly overcome the problems associated with partial tolerance to the tumor (self) antigen. Vaccine studies using DNA encoding whole protein or peptide fragments of HER-2/*neu* tumor specific antigen have been shown to be effective in augmenting the anti-tumor immune response.

Alphaviruses are positive strand RNA viruses that have been shown to infect and facilitate high-level gene expression in vertebrate cells. Recently, Alphavirus based expression systems have been developed which facilitate gene transfer *in vivo*. One such system is derived from the Venezuelan equine encephalitis virus (VEE). Using VEE, a non-replicative RNA replicon vector system has been established in which the viral structural genes have been removed and replaced by a foreign gene. Replicon RNA transcribed from plasmid DNA encoding an immunogen is packaged into infectious particles with the aid of helper RNA carrying the structural genes *in trans*. Deletion of the structural RNA from the replicon prevents the formation of progeny virus which may spread to other cells. This provides for a self-limiting infection which decreases the risk of inducing an immune response toward the vector. Consequently, the VEE replicon vector may be used for subsequent immunizations with the same or different immunogens.

The use of the VEE-based expression system for genetic vaccination has several advantages; foremost of which is that VEE preferentially infects DC *in vivo*. Dendritic cells (DC) are bone marrow derived cells known to be highly efficient antigen presenting cells for T cells. DC have the ability to present antigen in association with molecules of the major histocompatibility complex and provide potent co-stimulation in the form of cytokines and cell surface molecules such as CD80 (B7-1) and CD86 (B7-2). This costimulation is required for efficient activation of naïve T-cells and consequently, these cells are extremely effective at stimulating naïve $CD4^+$ and $CD8^+$ T-cells, and promoting effector cell differentiation. In addition to VEE's tropism for DC, the gene encoding an immunogen is under the control of a strong subgenomic 26S mRNA promoter resulting in high levels of expression. Finally, most human populations have had no previous exposure to VEE, and therefore no previous immunity that would initially limit expression of the immunogen.

The objective of this project has been to establish an effective tumor antigen specific immunotherapy directed against Her-2/*neu* proto-oncogene using VEE replicons.

Conclusions

Breast Cancer is currently the greatest public health concern and leading cause of cancer deaths among women. Most current therapies rely upon a combination of surgery, chemotherapy, radiation therapy and anti-estrogen therapy. In many cases, these treatments have proven to be painful and inadequate and often tumor recurrence is a problem. Our work has been focused on developing a safe and effective form of genetic vaccination to overcome immune unresponsiveness to the tumor associated antigen encoded by the Her-2/*neu* proto-oncogene. We have assembled an array of VEE replicons encoding the full-length Her-2/*neu* protein, a truncated version of Her-2/*neu* and several type-1 pro-inflammatory cytokines. Using combinations of these constructs, we have established the feasibility of enhancing Her-2/*neu*

specific immunity and preventing tumor progression. We have demonstrated the induction of Neu specific CTL activity using full length Her-2/*neu* and GM-CSF encoding replicons in wild type FVB mice. In order that we may directly monitor tumor progression, we have established a Her-2/*neu* expressing tumor cell line derived from FVB/*neu* mice. FVB/*neu* mice are permissive for growth of this cell line whereas wild type FVB are not. In our most encouraging experiments to date, vaccination with a VEE replicon encoding a truncated version of Her-2/*neu* has significantly delayed, and in some cases prevented the engraftment of tumor cells in FVB/*neu* mice. Current studies are ongoing to enhance the CTL response and evaluate the long term potential for preventing spontaneous tumor formation in FVB/*neu* transgenic mice. We believe a genetic vaccine approach using VEE replicons to target DC will result in an effective prophylactic for slowing the progression of established tumors and provide long-term protection from tumor recurrence.

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Overexpression and amplification of the Her-2/neu proto-oncogene has been implicated in the development of aggressive human breast cancer. Consequently, Her-2/neu provides a potential target for immunotherapy. We have employed a novel in vivo gene transfer system based on the Venezuelan Equine Encephalitis (VEE) virus. Our approach has been to establish VEE based replicons encoding Her-2/neu as well as pro-inflammatory cytokines known to promote CD4[+] Th1 and CD8[+] CTL activity. To determine the therapeutic efficacy of the VEE-based replicons, mice transgenic for the rat Her-2/neu gene (FVB/neu) have been employed. To date we have generated and tested replicons encoding the complete 185 kDa Her-2/neu transmembrane protein, a truncated protein spanning the extracellular and transmembrane domains of Her-2/neu (Neu/ECD-TM) as well as replicons encoding the cytokines IL-2, IL-4, IL-12 and GM-CSF. We have repeatedly generated CTL responses in wild type FVB mice and are currently evaluating strategies to overcome Her-2/neu-specific tolerance in FVB/neu mice. Additionally, we have established a Her-2/neu expressing tumor cell line derived from FVB/neu mice. FVB/neu mice are permissive for growth of the tumor cell line making this system amenable to tumor challenge experiments. We have observed delayed or absent tumor growth in FVB/neu mice immunized with the truncated VEE Her-2/neu construct and are currently evaluating the cellular phenotype responsible for this inhibition. We believe establishing a vaccine protocol using VEE replicons encoding Her-2/neu and appropriate cytokines will provide an effective strategy for enhancing tumor antigen-specific CD4[+] Th1 and CD8[+] CTL reactivity for the purpose of preventing tumor progression and providing long-term protection from tumor recurrence.

Tumor antigen-specific immunotherapy has long been thought of as an ideal approach to treating various forms of cancer. However, most tumor associated antigens are in fact self-antigens, and by definition poor immunogens. Consequently, the issue of self-tolerance remains a significant hurdle to overcome for inducing effective tumor-specific immunity. One such tumor-associated antigen is encoded by the Her-2/neu (erbB-2/neu) proto-oncogene. Her-2/neu encodes a transmembrane protein that is a member of the epidermal growth factor receptor (EGFR) family of proteins. Overexpression and amplification of Her-2/neu has been implicated in the development of aggressive breast adenocarcinoma as well as in the development of ovarian cancer. Cytolytic lymphocytes (CTL) cultured from human breast and ovarian cancer patients have been shown to be specific for a number of peptides derived from the Her-2/neu protein. This suggests tolerance to the self-antigen is incomplete and may provide for a window through which enhanced immune reactivity may be achieved. In FVB mice transgenic (Tg) for the rat Her-2/neu proto-oncogene (FVB/neu), 80% of females develop focal mammary tumors surrounded by hyperplastic mammary epithelium by 30 weeks of age. Of these, 70% display a tendency toward pulmonary metastasis by 8 months. Tumor cells removed from these mice display elevated levels of the transgene bolstering evidence for the oncogenic potential of Her-2/neu.

Vaccine studies using DNA encoding whole protein or peptide fragments of HER-2/neu tumor specific antigen have been shown to be effective in augmenting the anti-tumor immune response. Recently, Alphavirus based expression systems have been developed which facilitate gene transfer in vivo. One such system is derived from the Venezuelan equine encephalitis virus (VEE). Using VEE, a non-replicative RNA replicon vector system has been established in which the viral structural genes have been removed and replaced by a foreign gene. Replicon RNA transcribed from plasmid DNA encoding an immunogen is packaged into infectious particles with the aid of helper RNA carrying the structural genes in trans. Deletion of the structural RNA from the replicon prevents the formation of progeny virus which may spread to other cells. This provides for a self-limiting infection which decreases the risk of inducing an immune response toward the vector. The use of the VEE-based expression system for genetic vaccination has several advantages; foremost of which is that VEE preferentially infects DC in vivo. Dendritic cells (DC) are bone marrow derived cells known to be highly efficient antigen presenting cells for T cells. Additionally, the gene encoding an immunogen is under the control of a strong subgenomic 26S mRNA promoter resulting in high levels of expression. Finally, most human populations have had no previous exposure to VEE, and therefore no previous immunity that would initially limit expression of the immunogen.

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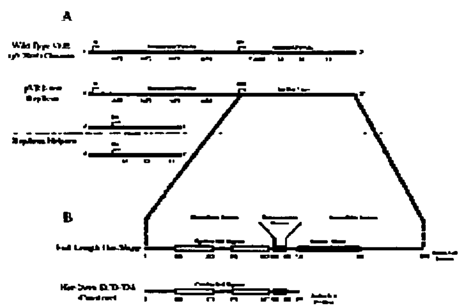
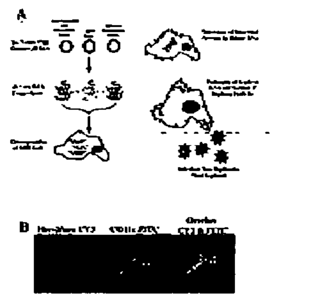
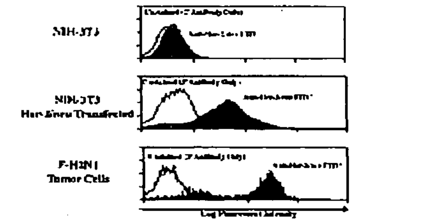
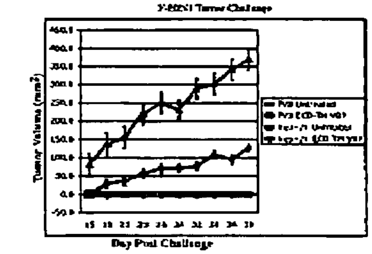


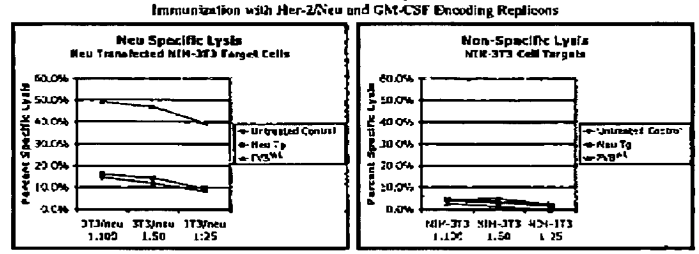






^{51}Cr release Assay

Immunization with Her-2/Neu and GM-CSF Encoding Replicons



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